Jurnal Kejuruteraan SI 2(1) 2019: 69-76
https://doi.org/10.17576/jkukm-2019-si2(1)-09

Polycaprolactone/Chlorophyllin Sodium Copper Salt Nanofibrous Mats Prepared by Electrospinning for Soft Tissue Engineering

(Gentian Nano Polikaprolakton/Klorofillin Natrium Kuprum melalui Elektroputaran bagi Aplikasi Kejuruteraan Tisu)

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Received 15 January 2019, Received in revised form 17 April 2019
Accepted 1 September 2019, Available online 30 December 2019

ABSTRACT

This study examined the process of synthesising biodegradable nanofibres made up of polycaprolactone (PCL) and chlorophyllin sodium copper (CSC) through electrospinning for scaffolding in tissue engineering. Scaffolds provide a platform for cell regeneration for repairing damaged human tissues or organs. However, the issue lies in developing scaffolding that will provide a favourable environment for cell attachment and proliferation. One way to address this concern is to add CSC, which has been widely used in biomaterial applications, to the nanofibres. The structure and morphology of the nanofibres in this research were determined by using a scanning electron microscope (SEM), and their chemical properties were tested by using Fourier-transform infrared spectroscopy (FTIR). Moreover, the diameter and adhesive force of the nanofibres were investigated by using an atomic force microscope (AFM). The SEM examination revealed that the PCL/CSC nanofibres lost their fibrous structure, and the FTIR results proved that the nanofibres synthesised by electrospinning still consisted of PCL and CSC. The AFM examination showed that the diameter and adhesive force of PCL/CSC nanofibres were less than those of PCL nanofibres. This outcome resulted from the CSC's inability to generate fibres on its own. Furthermore, its noncrystalloid structure prevented it from providing inner enhancement for PCL nanofibres. Hence, further studies are needed to ensure that PCL/CSC nanofibres can be used as an innovative type of scaffolding to provide an appropriate environment for living cells.

Keywords: Electrospinning; Biodegradable nanofibres; Polycaprolactone; Chlorophyllin; Tissue engineering

ABSTRAK

Objektif kajian ini adalah untuk mensintesiskan gentian nano terbiodegradasikan yang mengandungi polikaprolakton (PCL) dan klorofillin natrium kuprum (CSC) melalui elektroputaran bagi aplikasi kejuruteraan tisu. Kejuruteraan tisu memberikan terapi perubatan yang baru di mana ia menggunakan kelebihan yang terdapat pada biobahan polimer beserta pelopor hidup berbanding kaedah transplantasi konvensional. Tujuan kejuruteraan tisu adalah untuk membaiki tisu atau organ manusia yang rosak dengan menyediakan perancah bagi regenerasi sel. Walau bagaimanapun, masalah timbul apabila perancah yang dihasilkan hendaklah mempunyai persekitaran yang sesuai bagi pelekatan dan proliferasi sel. Oleh itu, penambahan CSC di dalam gentian mungkin mampu menyelesaikan masalah yang timbul kerana CSC telah digunakan secara meluas dalam aplikasi biobahan. Dalam ujikaji ini, struktur dan morfologi gentian ditentukan dengan mikroskopi elektron pengimbasan (SEM) dan ciri kimia gentian diuji dengan infra merah transformasian Fourier (FTIR). Diameter dan daya lekatan gentian dianalisa dengan mikroskopi daya atom. Pemeriksaan SEM menunjukkan gentian PCL/CSC mengalami kehilangan struktur asal gentian. Keputusan FTIR pula membuktikan gentian yang dihasilkan melalui elektroputaran masih mengandungi PCL dan CSC. Pemeriksaan AFM menunjukkan diameter dan daya lekat gentian PCL/CSC adalah kurang berbanding gentian PCL. Perkara ini mungkin disebabkan CSC tidak dapat menghasilkan gentiannya yang tersendiri dan struktur CSC tidak kristaloid yang tidak membenarkan sebarang peningkatan dalaman bagi struktur gentian. Justeru itu, penyelidikan lanjut perlu dilaksanakan bagi memastikan gentian PCL/CSC boleh dgunakan sebagai perancah yang berpotensi menyediakan persekitaran yang sesuai bagi sel hidup.

Kata kunci: Elektroputaran; Gentian nano terbiodegradasi; Polikaprolakton; Klorofillin; Kejuruteraan tisu

INTRODUCTION

In recent years, much attention has been given to nanofibers produced through electrospinning, which is a method that can produce fibres with uniform structures, create various types of polymers and is easy to use (Baji et al. 2010). This process uses an electrostatic force in which a strong repulsive electrical force is needed to overcome the surface tension of the charged polymer solution. Unlike other conventional spinning techniques, such as solution and melt spinning, which can only create fibres with diameters in the micrometre range, the electrospinning technique can produce fibres with diameters in the nanometre range. Moreover, fibres produced by electrospinning have their own benefits, including a high surface to volume ratio, tuneable porosity, malleability to conform to a wide variety of sizes and shapes and the ability to control the composition of the nanofibres to achieve the desired product (Bhardwaj & Kundu 2010). As such, these fibres have been successfully applied in various fields, such as tissue engineering. Tissue engineering represents an emerging interdisciplinary field that applies the principles of biological, chemical and engineering sciences for tissue regeneration. The main objective of tissue engineering is to provide scaffolds for cell regeneration to repair damaged human tissues or organs. Nanofibres have been explored as potential scaffolds for this purpose (Smith & Ma 2004) because their high porosity and surface areas have the potential to provide enhanced cell adhesion. Furthermore, the similarity between the nanofibre 3D architecture and natural extracellular matrix enables nanofibres to act as an excellent micro/nano environment for cells to grow and to perform their regular functions (Vasita & Katti 2006). For example, among their many uses, nanofibres can be applied to bone tissue engineering (Ye et al. 2019), cartilage tissue engineering (Cao et al. 2017), ligament tissue engineering (Rothrauff et al. 2017) and skin tissue engineering (Jiang et al. 2017).

Numerous factors can be manipulated during electrospinning to create scaffolds with different structural properties. These aspects include solution parameters, such as conductivity, concentration and viscosity and electrospinning parameters, such as the solution flow rate, the distance of the electric field generated and the electric field. Therefore, nanofibres produced through electrospinning have been strongly pursued as scaffolds for tissue engineering applications because their kinetic, mechanical and biological properties can be easily manipulated by altering the polymer solution composition and processing parameters (Qian et al. 2009). Single nozzle electrospinning is commonly used for tissue engineering applications owing to its ability to operate on a standalone basis or from a computer and its ease of use. Such electrospinning technique consists of a single set of nozzles connected to a high voltage power source. Recently, researchers have focused on the fabrication of tissue engineering scaffolds from a blend of two different polymers, namely, collagen/chitosan, polycaprolactone (PCL)/gelatin and PEO/collagen. This research involves the isolation of healthy cells from a patient followed by their expansion in vitro. The expanded cells are then seeded onto a three dimensional (3D) biodegradable scaffold that provides structural support and acts as a reservoir for bioactive molecules such as growth factors. For example, composites consisting of polyvinyl alcohol and chlorophyllin sodium copper (CSC) can regenerate skin dermal tissues and heal wounds (Jegina et al. 2016).

PCL is used in this study because of its nontoxic and biodegradable nature that offers flexible mechanical properties in terms of Young' modulus, tensile strength and elasticity (Dash & Konkimalla, 2012). PCL is mixed with other chemicals, such as CSC, to develop scaffolds and to provide a favourable environment for cell attachment and proliferation. CSC belongs to a group of water-soluble salts that is a semisynthetic derivative of chlorophyll. It is widely used as a food additive, scaffolding for tissue engineering and as treatment for medical conditions, such as wounds (Norman et al. 2016; Qian et al. 2009). Thus, CSC is used in this work to develop a scaffold that is suitable for tissue engineering. The objective of this research is to determine the structure, morphology, chemical content and diameter of the nanofibres as well as the strength of their adhesive forces. The structures and morphologies of the prepared nanofibres are investigated by using a scanning electron microscope (SEM), and their chemical properties are determined by using Fourier-transform infrared (FTIR) spectroscopy. The diameter and adhesive force of the nanofibres are determined by using an atomic force microscope (AFM).

METHODOLOGY

MATERIALS

PCL with a molecular weight of 80,000 g/mol, CSC and a phosphate buffer solution (PBS, pH = 7.2) were used in this experiment. The solvent agent used was 2,2,2-trifluroethanol (TFE). All materials were purchased from Sigma-Aldrich, and all reagents and materials were used as received without any further purification.

PREPARATION OF PCL/CSC NANOFIBRE MATS BY ELECTROSPINNING

Six samples with different PCL concentrations were dissolved in 100 ml of TFE and labelled, as shown in Table 1. Stirring was carried out for 1 hour at room temperature using a magnetic stirrer at a speed of 240 rpm. Then, 0.01 g of CSC was added into 3 polymer solution samples containing different concentrations of PCL, that is, 50 mg/mL, 100 mg/mL and 150 mg/mL. The remaining PCL solution samples were not mixed with the CSC because they were used for comparison. Each sample of the polymeric solution was stirred for 24 hours at room temperature using a magnetic stirrer. Each polymeric solution was then transferred into a 10 mL syringe.

The equipment used for electrospinning (ES-106) was purchased from the Nfiber Company. During electrospinning,

the flow rate of the solution was maintained at 1 mL/h for all samples. Applied voltage was maintained at 20 kV, and a 20-cm distance was maintained between the tip nozzles and the collector. This process was carried out for an hour at a temperature of 22 ± 5°C and a humidity of 35 ± 4%. Then, the samples were taken from the aluminium foil and dried for 12 hours at vacuum conditions.

TABLE 1. Six sample solutions prepared before the electrospinning process

Sample	Solution
1	50 mg/mL PCL
2	100 mg/mL PCL
3	150 mg/mL PCL
4	50 mg/mL PCL with CSC
5	100 mg/mL PCL with CSC
6	150 mg/mL PCL with CSC

MORPHOLOGICAL ANALYSIS

Square 5 mm^2 × 5 mm^2 strips were carefully cut from each electrospun fibre and coated with Pd for 45 seconds before being placed on the SEM under vacuum conditions. The distance between the edges of the electron gun with the sample surface was set at 7.2 mm, with the accelerating voltage of 15 kV. Magnification rates of 300 ×, 1000 × and 5000 × were used for testing the structural analysis and morphology for each nanofibre.

FTIR ANALYSIS

The sample was placed on the IR microscope plate for chemical analysis. The chemical content in the fibres were determined by using the available FTIR analysis software.

FIBRE DIAMETER AND ADHESIVE FORCE DETERMINATION

The samples were characterised using an atomic force miscroscope (AFM), and the images obtained from the characterisation tool were then analysed using an image processing software to determine the average diameter of each nanofibre. Measurements were taken at 50 different nanofibre locations from each picture by using the NOVA software. The adhesive force of the nanofibres were determined by performing force spectroscopy analysis on the sample using the AFM.

RESULTS AND DISCUSSION

MORPHOLOGY OF THE NANOFIBRES

During the electrospinning process, a polymer solution held by its surface tension at the end of a capillary tube was subjected to an electric field. An electric charge was induced on the liquid surface owing to this electric field. Repulsive electrical forces overcame the surface tension force when the electric field applied reached a critical value, thereby changing the spherical drop to a conical shape, which is known as the Taylor cone effect (De Pr et al. 2017). Eventually, a charged jet of the solution was ejected from the tip with an unstable and rapid whipping occurring in the space between the capillary tip and collector, which led to the evaporation of the solvent, thereby leaving a polymer behind (Bhardwaj & Kundu 2010) The polymer concentrations significantly affected the solidification process and thus influenced the morphologies of the electrospun nanofibres. The SEM analysis results of each sample are presented in Table 2

The structure of the formed nanofibres were smooth, cylindrical and beadless, with dense fibre networks with no particular alignment in the samples containing only the PCL. For the samples containing PCL/CSC, the nanofibres lost their basic morphology and structures, with some rupturing and forming tiny dense fibre networks with no particular alignment (Repanas & Glasmacher 2015). Therefore, the surface and structure of PCL nanofibres were smoother and more uniform compared to those of PCL/CSC nanofibres. Based on recent research, nanofibres containing PCL possessed uniform and smooth structures (Ren et al. 2017), whereas nanofibres containing PCL/CSC were neither uniform nor smooth (Qian et al. 2009).

FTIR-ATR SPECTRA OF THE ELECTROSPUN NANOFIBRE MATS

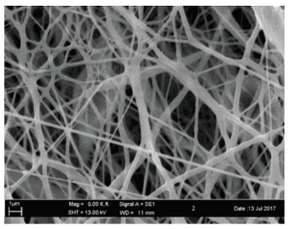
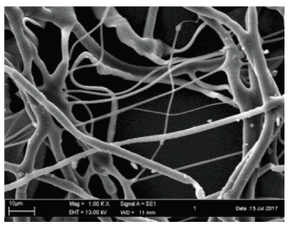
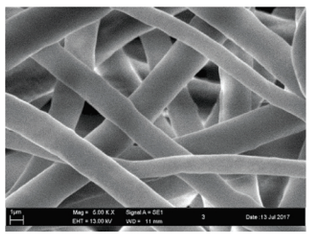
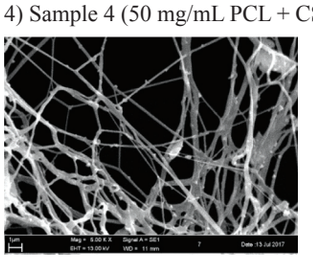
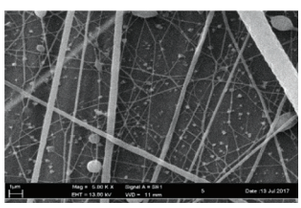
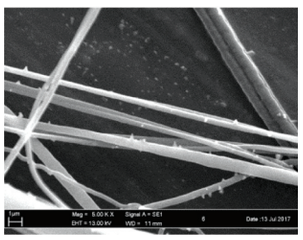
The FTIR analysis results of each sample are presented in Figures 1–6. The spectrum shows major stretching regions between several wavelengths for all the samples. The first analysis on the peak involved determining the presence of significant functional groups of PCL. The carbonyl group of C = O was significantly present between wave numbers 1,500 to 1,800 cm^{-1}. The presence of the carbonyl group indicated a strong absorption because the peak for C = O was the strongest in the spectrum and had a medium width (Elzein et al. 2004). According to Coleman and Zarian, the peak at 1,294 cm^{-1} was assigned to the backbone C-C and C-O stretching modes in the PCL. All samples showed the presence of backbone C-C and C-O stretching modes in which the peak appeared at approximately 1,294 cm^{-1} (Coleman & Zarian, 1979). The bands and their assignments presented in Table 3 prove that all the nanofibre samples contained PCL.

Absorption bands were observed in the range of 3,600 – 2,850 cm^{-1} for Samples 4, 5 and 6, which contain a combination of CSC and PCL nanofibers. This outcome is attributable to the stretching vibrations of O-H, N-H and C-H bonds.

TABLE 3. Characteristic PCL IR bands

Frequency (cm^{-1})	Type of Vibration
2,926	Asymmetric CH_2 stretching
2,853	Symmetric CH_2 stretching
1,724	Carbonyl stretching, C = O
1,294	C-O and C-C stretching
1,240	Asymmetric COC stretching

TABLE 2. Nanofibre structure and morphology for each sample

Sample	Observation
1) Sample 1 (50 mg/mL PCL) 	• Creation of smooth, cylindrical and beadlessnanofibres • Formation of dense fibre networks with no particular alignment • Formation of uniform-sized nanofibres
2) Sample 2 (100 mg/mL PCL) 	• Most nanofibres formed were smooth and cylindrical • Beaded droplets formed on the surface and structure of the nanofibres
3) Sample 3 (150 mg/mL PCL) 	• Creation of smooth, cylindrical and beadlessnanofibres • Formation of dense fibre networks with no particular alignment • Formation of big and uniform nanofibres
4) Sample 4 (50 mg/mL PCL + CSC) 	• Creation of smooth, cylindrical and beadlessnanofibres • Nanofibres contained a liquid layer with droplets of beads forming on their surfaces
5) Sample 5 (100 mg/mL + CSC) 	• Creation of smooth and cylindrical nanofibres • Formation of dense fibre networks with no particular alignment • Formation of tiny dense fibre networks with no particular alignment from ruptured fibres • Formation of bead droplets around the nanofibres
6) Sample 6 (150 mg/mL + CSC) 	• Creation of smooth and cylindrical nanofibres • Formation of dense fibre networks with no particular alignment • Formation of bead dropletson the surface of the nanofibres

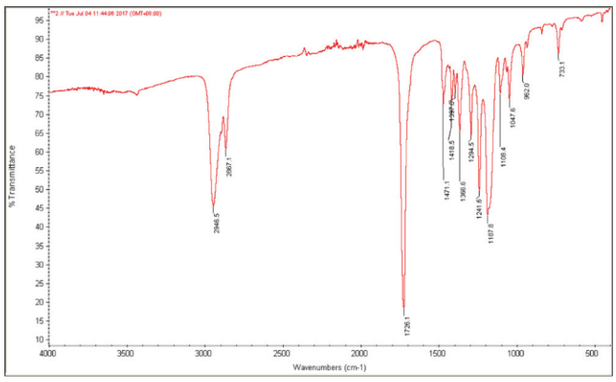


FIGURE 1. FTIR analysis for Sample 1

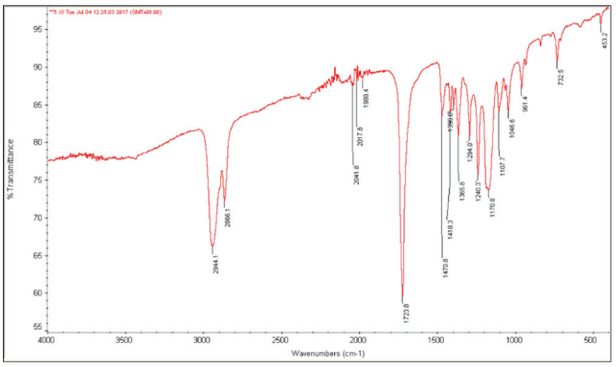


FIGURE 5. FTIR analysis for Sample 5

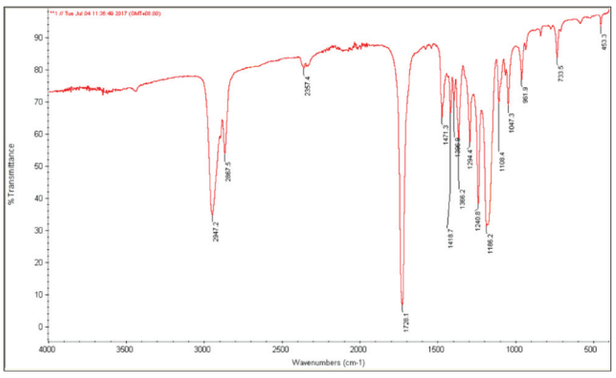


FIGURE 2. FTIR analysis for Sample 2

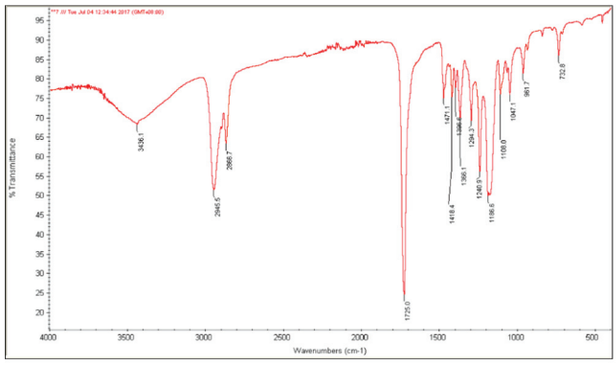


FIGURE 6. FTIR analysis for Sample 6

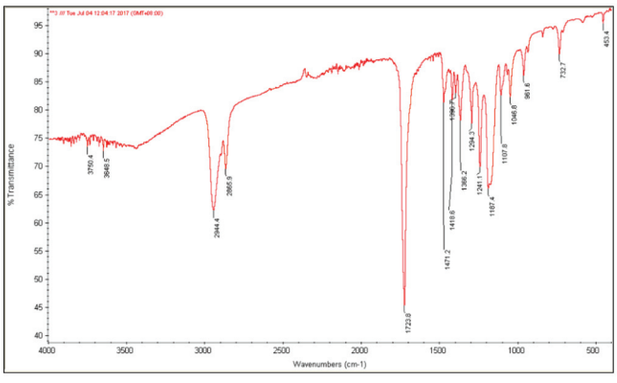


FIGURE 3. FTIR analysis for Sample 3

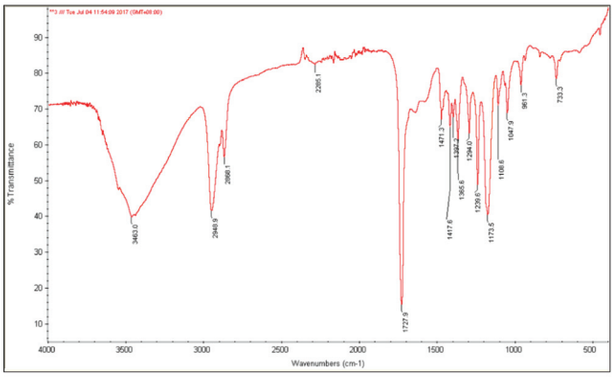


FIGURE 4. FTIR analysis for Sample 4

The signal with a maximum intensity in 3,436 cm^{-1} was assigned to the stretching vibration of the OH groups in water molecules, which occurred only in Samples 4 and 6. Based on theory, Sample 5 should show the same peak as Samples 4 and 6 because CSC is a water-soluble salt. This outcome may due to the small CSC quantity used, which weakened the produced peak and rendered the FTIR unable to detect the presence of the stretching vibrations of OH groups in the nanofibres for Sample 5. Moreover, the presence of aromatic rings in the structure of the CSC was observed in the range of 1,475 – 1,600 cm^{-1} (Norman et al. 2016). However, according to the results, weak peaks were produced rather than a strong intensity because the main structure of the CSC consisted of aromatic rings. Again, this error occurred because of the small CSC quantity used, which made it difficult for the FTIR to detect the presence of aromatic rings. Finally, the peak which occurred approximately 700 cm^{-1} increased from the vibration of the metal atom (copper) present in the structure of the CSC, thereby indicating the presence of CSC in the PCL nanofibres.

DIAMETER AND ADHESIVE FORCE OF THE NANOFIBRES

The diameter and adhesive force of the nanofibres represent important properties that must be considered for tissue engineering scaffolds (Xing et al. 2010). This circumstance is because the scaffolds must be strong enough to resist force from body movements or from the outer environment and provide enough space for living cells (Lee et al. 2017).

Table 4 shows the average diameter of the nanofibres for each sample, with measurements taken at 50 different locations in each picture using the NOVA software.

TABLE 4. Average diameter of nanofibres and PDI for each sample

Sample	Average diameter of nanofibre, x (nm)	Polydispersity (PDI)
1	774.8 ± 280.5	0.13
2	1016.6 ± 453.2	0.20
3	1769.8 ± 474.7	0.07
4	764.8 ± 247.2	0.10
5	603.4 ± 207.6	0.12
6	632.6 ± 290.3	0.21

As shown in Table 4, the diameter of the nanofibres produced in Samples 4, 5 and 6, which contain a combination of CSC and PCL nanofibers, are smaller than those of samples with only PCL nanofibers. The presence of CSC changes the basic morphology of nanofibres in which some fibres rupture and form tiny dense fibre networks with no particular alignment (Qian et al. 2009). The loss of parts of the nanofibrous structure provides enough space for cells to move into the inner sections of the nanofibrous mats because without the presence of CSC, the PCL nanofibrous mats alone are too dense for cells to migrate inside the scaffold.

During contact modes in the AFM, adhesion forces have significant effects on the cantilever during probe withdrawal from the sample. The forces result in the deflection of the cantilever before it breaks contact with the surface. The DFL signal first falls below its observed value well away from the surface with the reduction of the z-scanner length and then abruptly reaches the free-state value, thus forming a specific dip.

The adhesion force can be calculated by assuming that the force is a linear function of the probe displacement relative to the sample surface along the Z-axis. The AFM analysis showed the DFL (height) curve in a point of each sample (Figures 7–12). According to Hooke's law:

$$F = k \times \Delta\, Height \qquad (1)$$

where k is the cantilever stiffness. In this study, the cantilever stiffness k was 0.5 N/m, whilst the value of height Dx was obtained by using the NOVA software. For example, on the NOVA software, the 'pair markers' button was depressed and the mouse was used to set the two markers on the section of the curve containing a slope.

As shown in the results in Table 5, the adhesion force of PCL without CSC nanofibres is higher than that of nanofibres containing a combination of PCL and CSC. Adding CSC in nanofibres had no positive effects on the adhesion force of PCL nanofibres and in fact weakened them. This decrease of adhesion force despite the presence of CSC was because the PCL/CSC nanofibers used were a simple blend of PCL and CSC. Moreover, no chemical reaction occurred between the PCL and the CSC because CSC cannot generate any fibres on its own, and its crystalloid structure does not allow it to act as an inner enhancement for the PCL nanofibres (Qian et al. 2009). This decrease in adhesion force rendered the PCL/CSC nanofibres unsuitable for scaffolding in tissue engineering because the amount of CSC used in this study may be inadequate for the formation of PCL/CSC nanofibres. Therefore, the right amount of CSC must be added into the PCL nanofibres to create a scaffold with high adhesion force and to simultaneously provide enough space for cells to move into the inner section of the nanofibrous material.

TABLE 5. Adhesive force of nanofibres for each sample

Sample	Height, Dx (nm)	Adhesion force, F (Nn)
1	86	43
2	90	45
3	138	69
4	80	40
5	54	27
6	78	39

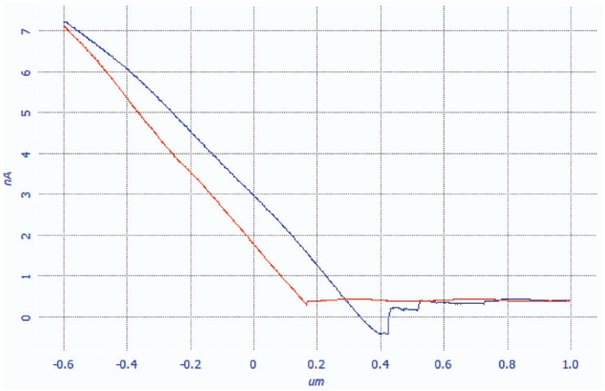


FIGURE 7. DFL (height) curve in a point for Sample 1

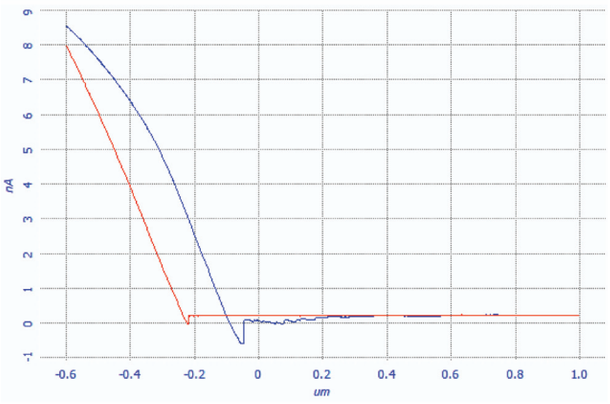


FIGURE 8. DFL (height) curve in a point for Sample 2

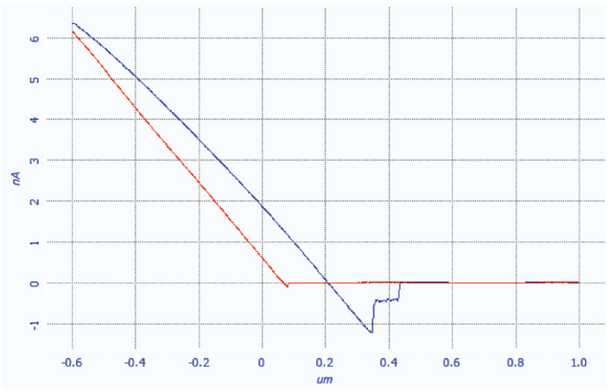


FIGURE 9. DFL (height) curve in a point for Sample 3

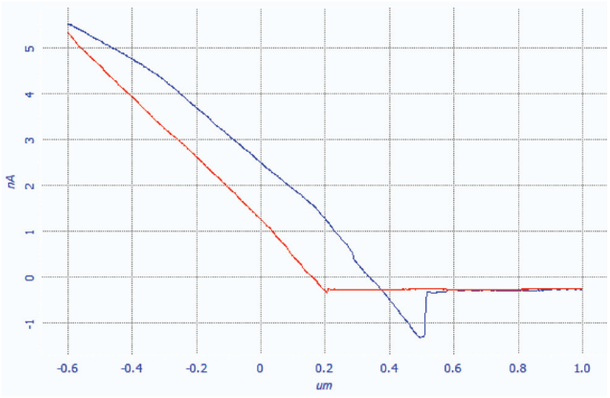


FIGURE 10. DFL (height) curve in a point for Sample 4

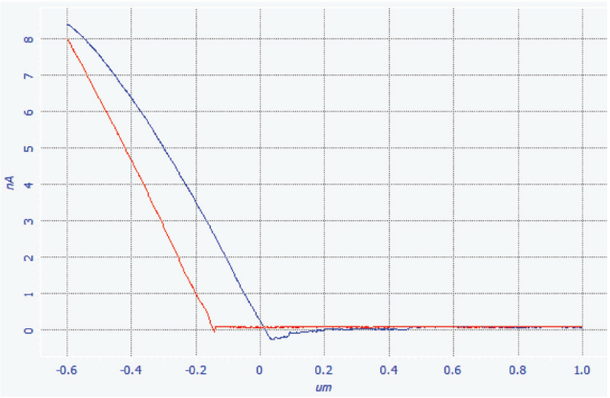


FIGURE 11. DFL (height) curve in a point for Sample 5

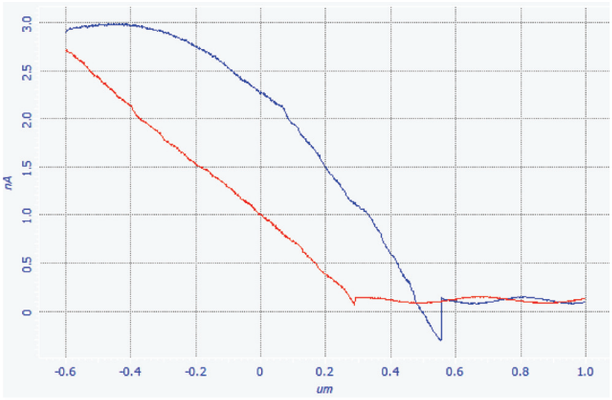


FIGURE 12. DFL (height) curve in a point for Sample 6

CONCLUSION

PCL/CSC nanofibres were successfully prepared by electrospinning the TFE solution. This research was performed to study the effects of CSC content on the morphology, structure and diameter of nanofibres. The presence of CSC changed the basic morphology of nanofibres by rupturing fibres and forming extremely small, dense fibre networks with no particular alignment. This outcome showed that the presence of CSC in PCL nanofibres may provide additional space for cells. For this research, PCLalone was a better option for tissue engineering applications because it provided a higher adhesion force compared to the PCL/CSC nanofibers. Moreover, the scaffold must be strong enough to resist force from body movements or from the outer environment. However, CSC is still needed because it provides adequate space for living cells in the scaffold. Furthermore, nanofibrous mats are too dense to allow cells to migrate inside the scaffold. As such, further study is required to determine the optimum amount of CSC to be added into PCL nanofibrous mats. This additional investigation would ensure the utilisation of PCL/CSC nanofibres as an innovative type of scaffolding to provide a favourable environment for living cells.

ACKNOWLEDGMENT

The authors with to express their appreciation to Universiti Kebangsaan Malaysia and to the Ministry of Education Malaysia for funding this research work through the GUP-2017-102 grant.

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